EXHIBIT A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Riviere *et al.*

Serial No.: 08/252,710 Group Art Unit: 1805

Filed: June 2, 1994 Examiner: G. Elliott

For: **RETROVIRAL VECTORS USEFUL FOR GENE THERAPY** Attorney Docket No.: 8141-113-999

AMENDMENT UNDER 37 C.F.R. § 1.116

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Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

The Applicants acknowledge the receipt of the Office Action mailed on April 17, 1996 (Paper No. 12) which has been carefully reviewed and studied. The Applicants respectfully request that the Examiner enter the following amendments to the specification and claims, and reconsider the application in view of the following remarks. In order to facilitate the Examiner's evaluation of the application, the Applicants have attempted to address the Examiner's rejections in the same order in which they were raised in Paper No. 12.

Please amend the application as follows:

Please replace pages 10 and 11 of the specification with the attached substitute pages 10 and 11. The particular

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EXPRESS MAIL CERTIFICATION

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Gabriel Urquidez
(Type or print name of person mailing paper or fee)

(Signature of person mailing paper or fee)

corrections made in the substitute pages are discussed below. None of the changes are deemed to constitute new matter.

SUMMARY OF THE INVENTION

The present invention is directed to a novel class of retroviral vectors that have been engineered to be capable of transducing mammalian cells at such high efficiency that no selection step is required (when used with the appropriate packaging cells lines). Given that no selection is required, the presently described retroviral vectors do not encode a functional selectable marker. This feature of the present vectors renders them novel over all prior art retroviral vectors, and provides the additional advantage that present vectors may incorporate larger inserted genes than previous retroviral vectors.

REMARKS

I. Provisional Double Patenting Rejections

The Applicants gratefully acknowledge the Examiner's withdrawal of the rejection of claims 5, 6, and 15 under 35 U.S.C. §§ 101 and 102(e) over U.S. application Ser. No. 07/786,015, now abandoned.

The Applicants request that the Examiner postpone further consideration of the provisional obviousness-type rejection of claims 1 through 4 and 6 through 8 over U.S. application Ser. No. 08/486,858 until claims in either application have been deemed allowable.

II. Rejections based on prior art.

A. Rejections under 35 U.S.C. § 103 in view Cone and Mulligan in conjunction with Temin and Bender *et al.*

The Examiner has rejected Claims 1-4, 6-8, 20, 21, and 35-37 as obvious over the teaching of Temin in view of Bender *et al.* (Bender) and Cone and Mulligan (Cone). The Examiner's rejection of the claims based on 35 U.S.C. § 103 over the combined teaching of Temin, Bender, and Cone is respectfully traversed. Neither Temin nor Bender teach or suggest recombinant retroviral vectors that are capable of transducing mammalian cells without selection. Accordingly, Temin and Bender cannot be combined to teach recombinant retroviral vectors which lack a complete selectable marker.

Cone is ostensibly cited because it contains a comment that titers of >$\underline{10^5}$ virus per ml are high enough to enable the nonselective introduction of genes into 100% of a population of cells. However, Cone merely suggested that titers of approximately 10^5 virus per ml were "...high enough to facilitate the nonselective introduction of genes into 100% of a population of cells...". In fact, such titers are woefully inadequate for the stated purpose as evidenced by the fact that the Examiner has cited <u>no</u> prior art references teaching retroviral transduction without selection.

Where the prior art, as a whole, does not explicitly suggest the claimed invention, a proper obviousness analysis under §103 requires consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should carry out the claimed process; and (2) whether the prior art would have also revealed that in so

carrying out, those of ordinary skill would have a reasonable expectation of success, *In re Vaeck*, 947 F.2d 488, 493 (Fed.Cir.1991). Thus, given Cone's "suggestion", a proper *prima facie* obviousness rejection requires that the Examiner establish that the teaching provided by the combined references would have provided a reasonable expectation of success. However, in spite of the fact that <u>seven</u> years had elapsed between Cone's alleged suggestion, and the effective filing date of the present application, the Examiner has cited <u>no</u> references that affirmatively demonstrate the construction and operability of retroviral vectors that lack a selectable marker. In other words, <u>the Examiner has provided no evidence supporting the reasonable expectation of success</u>.

One of the legally recognized considerations of nonobviousness is the "failure of others" to construct the claimed invention (*Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, (Fed. Cir. 1986). The Examiner is respectfully requested to consider that the level of ordinary skill in the art is very high in the field of biotechnology, and that the pace of innovation is equally impressive. The Applicants submit that the absence of any reference teaching the successful implementation of Cone's allegedly enabling suggestion during the <u>seven</u> years prior to the effective filing date of the present application constitutes clear and convincing evidence that the claimed invention is *per se* nonobvious over the art of record. In fact, the demonstrated "failure of others" is compelling evidence of nonobviousness that effectively rebuts the alleged *prima facie* case of obviousness raised by the Examiner.

In view of the Applicants' clear rebuttal of the Examiner's *prima facie* case of obviousness, it is incumbent upon the Examiner to present evidence that the teaching of Cone would have provided a reasonable expectation of successfully constructing and practicing the claimed retroviral vectors. Presently, the Examiner has provided no evidence that the cited art of record would have provided one of ordinary skill with a reasonable expectation of success. Lacking such evidence, the Examiner has not established the legal basis for a proper obviousness rejection. Accordingly, the Examiner is respectfully requested to reconsider and withdraw rejections under 35 U.S.C. § 103 over the teaching of Cone.

As further support of the above position, the Applicants are submitting herewith the Declaration of Dr. Lawrence Cohen. In his Declaration Dr. Cohen states that it is his expert opinion that the concentrations of retrovirus disclosed by Cone would have not provided one of ordinary skill with a reasonable expectation of successfully transduce mammalian cells without selection. Dr. Cohen provides supporting evidence for his opinion in the form of personal statements, and a reference which shows that one must generally use a concentration of retrovirus that is at least about an order of magnitude higher than that taught by Cone in order to practice efficient transduction without selection.

In brief, Dr. Cohen's Declaration provides additional proof that Cone's unsupported and conclusory statement was wrong. Accordingly, the Examiner's reliance on Cone is also wrong. Given that an inoperative combination can not provide

one of ordinary skill with teaching sufficient to provide a reasonable expectation of success, the Applicants respectfully request that the Examiner further reconsider and withdraw the rejection of the pending claims as obvious over Cone.

The Examiner is also respectfully requested to consider that, especially where the transduced mammalian cells are to be used *in vivo*, it is generally desirable to minimize the cell's expression of foreign gene products. For example, the *neo* gene product is derived from a bacterial transposon, and is thus foreign to the body. Given that the presence of foreign proteins in the body may be expected to elicit an immune response against the transduced cells, it is clear that many *in vivo* applications of transduced cells mitigate against the presence and expression of a selectable marker. None of the cited prior art even mention this problem, and thus it is impossible for the Examiner to now argue that the cited references somehow teach or suggest the solution. In fact, in view of the above considerations, it should be clear that references teaching retroviral vectors that incorporate and express a functional selectable marker actually teach away from the invention as presently claimed.

Case law clearly holds that non-obviousness can lie in the discovery of a problem, the solution to which employs the combination of old elements, see, *In re Sponnoble*, 160 U.S.P.Q. 237 (CCPA 1969). Moreover, the non-obviousness involved in the discovery of the reason for the problem can impart patentability to the solution thereto, even though by hindsight the cause of the problem once recognized may suggest the solution. *In re Leonnart and Espy*, 135 U.S.P.Q. 307 (CCPA

1962), *Trio Process Corp. v. L. Goldsteins Sons, Inc.*, 174 U.S.P.Q. 129 (CA3 1972), *In re Roberts et al.*, 176 U.S.P.Q. 313 (CCPA 1973), *In re Nomiya et al.*, 184 U.S.P.Q. 607 (CCPA 1975), *Ex parte Campbell et al.*, 211 U.S.P.Q. 575 (POBA 1980). Additionally, the Examiner is directed to *In re Peehs and Hunner*, 204 U.S.P.Q. 835 (CCPA 1980), where the Court stated:

> Where the Applicant contends that the discovery of the source of a problem would have been unobvious [non-obvious] to one of ordinary skill in the pertinent art at the time the claimed invention was made, it is incumbent upon the PTO to explain its reasons if it disagrees. A mere conclusionary statement that the source of a problem would have been discovered is inadequate. As this court explained in *In re Sponnoble*, 56 CCPA 823, 832, 405 F.2d 578, 585, 160 USPQ 237, 243 (1969): "A patentable invention may lie in the discovery of the source of a problem even though the remedy may be obvious once the source of the problem is identified. This is part of the 'subject matter as a whole' which should always be considered in determining the obviousness of an invention under 35 U.S.C. § 103."

Simply put, none of the cited publications, alone or in combination, explicitly suggest that it may be desirable to construct retroviral vectors which do not encode complete selectable markers. Thus, it is axiomatic that since none of the cited references even recognized the problem, the references, alone or in combination, could not have motivated or suggested the solution to one of ordinary skill in the art. Absent such motivation or suggestion, the cited art cannot properly support a *prima facie* case of obviousness under 35 U.S.C. § 103.

Given the above perspective, the Applicants respectfully submit that the presently claimed vectors (which do not encode or express a complete selectable marker) are *per se* nonobvious, and, accordingly, the Examiner is respectfully

requested to withdraw the rejections of claims 2-6, 9-20, and 22-31 under 35 U.S.C. § 103.

The Examiner has also rejected claims 2-4 and 20 as unpatentable under 35 U.S.C. § 103 as obvious over the combined teachings of Temin, Cone, and Bender. The Applicants respectfully traverse the rejection for the reasons stated above regarding the inadequacies of the Cone reference.

The Examiner has also rejected claims 9 and 20 as unpatentable under 35 U.S.C. § 103 as obvious over the combined teachings of Temin, and Cone, in further view of Kenten *et al.* (Kenten), or Kuo *et al.* (Kuo). The Applicants note neither Kenten nor Kuo teach or suggest retroviral vectors that lack selectable markers. Thus, the teaching of Cone is central to the Examiner's rejection, and the Applicants respectfully traverse the rejection for the reasons stated above regarding the inadequacies of the Cone reference.

The Examiner has also rejected claims 10, 11, 17, 18, and 20 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, and Cone, in further view of Emerman *et al.* (Emerman). Since Emerman does not teach or suggest the construction or use of retroviral vectors that lack a selectable marker, the teaching of Cone is also the center-piece of this rejection. The Applicants respectfully traverse the rejection for the reasons stated above.

The Examiner has also rejected claims 16 and 20 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, and Cone, in further view of Emerman, and Yee *et al.* (Yee) or Yu *et al.* (Yu). Since none of Yee, Yu, or Emerman teach or suggest the construction or use of retroviral vectors

that lack a selectable marker, the teaching of Cone remains key to this rejection. Given that the teaching of Cone is inoperative, the Applicants respectfully traverse the rejection for the reasons stated above.

The Examiner has also rejected claim 19 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, and Cone, in further view of Emerman, Yee, or Yu. Since none of Yee, Yu, or Emerman teach or suggest the construction or use of retroviral vectors that lack a selectable marker, the teaching of Cone is apparently the hub of this rejection. Given that the teaching of Cone is inoperative, the Applicants respectfully traverse the rejection for the reasons stated above.

The Examiner has also rejected claims 12-15, 20 and 22 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, Cone, and Emerman in further view of Anderson and deVilliers. As discussed above, none of Temin, Emerman, Anderson, or deVilliers teach or suggest the construction or use of, or even a motivation for, retroviral vectors that do not encode a functional selectable marker. Accordingly, the Examiner is respectfully requested to withdraw this rejection in view of the previously discussed inadequacies of the teaching of Cone.

The Examiner has also rejected claims 23 and 24 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, Cone, Anderson, and deVilliers in further view of Hilberg *et al.* (Hilberg) or Holland *et al.* (Holland). None of Temin, Anderson, deVilliers, Hilberg, or Holland teach or suggest the construction or use of, or even a motivation for,

retroviral vectors that do not encode a functional selectable marker. Accordingly, the Examiner is respectfully requested to withdraw this rejection in view of the previously discussed inadequacies of the teaching of Cone.

The Examiner has also rejected claims 25-31 as obvious under 35 U.S.C. § 103 over the combined teachings of Temin, Cone, Anderson, and deVilliers taken with Hilberg or Holland, or in further view of Franz *et al.* (Franz). None of Temin, Anderson, deVilliers, Hilberg, Holland, or Franz teach or suggest the construction of, use of, or even a motivation for, retroviral vectors that do not encode a functional selectable marker. Accordingly, the Examiner is respectfully requested to withdraw this rejection in view of the previously discussed inadequacies of the teaching of Cone.

III. Rejections under 35 U.S.C. § 112

The Examiner has rejected claims 1-31 and 35-37 because of certain objections to the specification for failing to provide an adequate written description of the invention. In particular, the Examiner has cited the specification at page 10, lines 27-28 where a typographical error resulted in several unlabeled boxes which correspond to regions of Fig. 11.

As requested by the Examiner, the Applicants are submitting substitute pages 10 and 11 that contain the corrections requested by the Examiner. In particular, the shading corresponding to the ADA cDNA (which is clearly labeled in Fig. 11(A)); and the shaded regions corresponding to MPSV and Friend sequences (which are also clearly and

unambiguously shown in Fig. 11(A), e.g., "MPSV-LTR"). Further support for the shaded MPSV sequence may be found at page 10, lines 12-16 where the specification describes the 385 bp *Nhe I-Sac I* fragment containing the MPSV enhancer that is clearly marked in Fig. 11(A) (note: the "*Nhe I-Sac I*" fragment of MPSV DNA shown in the "MPSV-Enh" construct). Similarly, support for the shaded regions corresponding to Friend sequences are clearly and unambiguously shown in Fig. 11(A) and are described in the specification at page 10, lines 22-25 (note: the "*Nhe I-Kpn I*" fragment (of Friend sequence from pFr-SV) that is clearly marked in Fig. 11(A). The applicants have also removed the empty boxes ("(□)") from substitute page 10, at line 34, and substitute page 11, at line 1 because of the specification's description of an ADA expressing variant of α-SGC is deemed to be self-explanatory (especially in view of the detailed structure of α-SGC presented in Fig. 4 and the clearly shaded region of ADA sequence shown in Fig. 11). While preparing substitute page 11, the Applicants also corrected the following typographical errors:

1) Line 10, "copy" was replaced with --copies--.
2) Line 12, "correspond" was replaced with --corresponding--.
3) Line 27, "activity control" was replaced with --activity. Control--.

In view of the above comments, the corrected pages are not deemed to constitute new matter. In view of the above corrections to the specification, the Examiner's rejection of claims 1-31 and 35-37 under 35 U.S.C. § 112, first paragraph is deemed to have been avoided by amendment.

CONCLUSION

In view of the foregoing amendments and remarks, the Applicants believe that the application is in good and proper condition for allowance. Early notification to that effect is earnestly solicited. If the Examiner feels that a telephone call would expedite the consideration of the application, the Examiner is invited to call the undersigned attorney at (415) 926-7405. The Commissioner is authorized to charge any underpayment or credit any overpayment to the deposit account number 16-1150 for any matter in connection with this response, including any fee for extension of time which may be required.

Respectfully submitted,

PENNIE & EDMONDS

Dated: 10/15/96 By: Albert P. Halluin 25,227

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the env mRNA. A point mutation (A-C) has converted the *Nla III* site into an *Nco* I site encompassing the env initiation codon where the human ADA coding sequence was inserted (from *Nco* I +74 to Acc I +1324 within the huADA cDNA (▩). Daddona, P.E., et al., J. Biol. Chem. 259:12101-12106, (1984). The Mo LTR/B2 vector was constructed by ligating the 1274 bp *Hind* III-Pvu I fragment of the PEM-ADA vector, Wilson, J.M. et al., Proc. Natl. Acad. Sci. USA 87:439-443, (1990), that contains the B2 mutation (G to A at position +160) to the *Hind* III-Pvu I fragment of MFG. The MPSV enhancer was cloned into MFG by replacing the *Nhe I-Sac* I fragment of the 3' Mo MuLV LTR with the 385 bp corresponding fragment from the 3' MPSV-LTR plasmid (kindly provided by P. Robbins, Pittsburgh, PA) to generate the MPSV-Enh construct. The MPSVE-EnhB2 was analogously derived from MPSV-Enh and PEM-ADA constructs. In the MPSV-LTR construct, the 6014 bp *Ban II-Nhe* I fragment from the pC663neoR plasmid, Ostertag, W., et al., J. gen. Virol. 67:1361-1371, (1986), has been replaced with the 2694 bp *Ban II-Nhe* I fragment from the MFG vector. To generate the Fr-Enh construct, the 450 bp *Nhe I-Kpn* I fragment of MFG was replaced with the corresponding *Nhe I-Kpn* I fragment from the pFr-SV (X) plasmid, Holland, et al., Proc. Natl. Acad.Sci., USA, 84:8662-8666, (1987).

Mo-MuLV LTR (□), MPSV sequences (▨), Friend sequences (▩).

(B) α-SGC vector: The α-SGC vector derived from pHSG, bears a portion of *gag* and an enhancer deletion in the 3' LTR, Guild, et al., J. Virol., 62:3795-3801, (1988). In this vector, huADA expression is under the control of the human cytomegalovirus (CMV) enhancer (□) (*Spe*I +154-*Nco* I +515 fragment), Boshart, M. et al., Cell, 41:521-530,

C'

C

(1985), and α-globin promoter (*Pst 1* -570 -*Nco*I +37 fragment), Braelle, F.E., Cell, 12:1085-1095, (1977).

(C) DNA analysis of NIH 3T3 cells infected with the recombinant retroviruses: After infection of NIH 3T3 cells under standard conditions (see Section 11.1, *infra*), genomic DNA was digested with *Nhe I* and analyzed by Southern blot using a huADA cDNA probe. Each lane was loaded with 10 μg of genomic DNA. The number of proviral copies per cell is indicated under each lane as determined with the Phosphorimager. In the left lane, the copy control corresponding to 1 copy per cell of the Mo-LTR vector.

Figure 12. Analysis of human ADA expression in peripheral blood cells:

(A) Analysis of hADA expression 5-7 months after BMT. The time at which blood samples were drawn is indicated in days after transplantation for each vector. hADA activity was measured by IEF (see Section 11.1., *infra*). The number directly above each sample indicates individual animals. The number of cells injected in every recipient is indicated above and extends from 2×10^5 to 4.5×10^6 cells. The lower band on the gel represents the activity of the murine endogenous ADA (mADA) and the upper band represents the human ADA (huADA) activity. Control samples were prepared from non-transplanted mice. The italic numbers indicate the mice which were examined in detail in Figure 13.

(B) Fraction of mice expressing huADA at 5-7 months after BMT. Relative ADA activity (r) represents the ratio of the intensity of human to mouse ADA enzyme bands determined on Figure 12A: with the computer densitometer. n_1 indicates the number of

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Mulligan *et al.*

Serial No.: 08/252,710	Group Art Unit: 1805
Filed: June 2, 1994	Examiner: G. Elliott
For: RETROVIRAL VECTORS USEFUL FOR GENE THERAPY	Attorney Docket No.: 8141-113

DECLARATION UNDER 37 C.F.R §1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Lawrence Cohen, declare and state that:

1. I am an employed by Somatix Therapy Corporation as Vice President of Research. I have worked at Somatix Therapy Corporation since 1988. My Curriculum Vitae is presented herewith as Exhibit 1.

2. I am familiar with the Specification and Claims of the above-identified patent application (heretofore the "present application") and the Office Action mailed April 17, 1996 (Paper No. 12).

3. It is my understanding that the Examiner contends that the Claims of above-identified application are obvious over the teaching of Cone and Mulligan, 1984

(Proc. Natl. Acad. Sci. *81*:6349-6353) in combination with other references.

4. I have read and am familiar with the above-identified reference by Cone and Mulligan ("Cone").

5. It is my opinion that, given the teaching in the Cone reference, one of ordinary skill in the art as of the filing date of the present application would not have had a reasonable expectation of successfully practicing the claims of the present application. My opinion is based on the fact that subsequent studies have shown that retroviral titers of the concentration disclosed by Cone (">10^5") are insufficient to effectively transduce mammalian cells without selection. For example, to my knowledge the first published report that primary human tumor cells could be transduced without selection was made in 1993 by Jaffee *et al.*, 1993, Cancer Research *53*:2221-2226 ("Jaffee", attached as Exhibit 2). Jaffee used a novel retroviral vector that is an embodiment of the vectors described in the above-identified application. To my knowledge, retroviral transduction without selection (as reported in Figure 1 of Exhibit 2) generally requires high titer stocks of transducing virus. In particular, a <u>minimum</u> titer of approximately <u>5 x 10^6</u> transducing virus per ml is required. This concentration of retrovirus is at least several fold higher (if not a full order of magnitude higher) than the retroviral titers reported by Cone. Accordingly, it is

my opinion that one of ordinary skill could not have used the teaching of Cone to practice the presently claimed invention.

6. It is also my opinion that, prior to the present invention, the cell lines and vectors taught by Cone would have not provided one of ordinary skill with a general expectation that mammalian cells could be successfully transduced without the use of selectable markers. In fact, the first published disclosure of human primary tumor cells transduced without selection (i.e., Jaffee *et al.*) was made nine years after Cone was published. During those nine years, novel retroviral packaging cell lines were constructed (as described in U.S. Patent No. 5,449,614 "'614", issued September 12, 1995), and the equally novel retroviral vectors of the present application were constructed. Jaffee *et al.* obtained these pioneering results after combining novel vectors and a packaging cell line that were both developed well after Cone was published. It should also be noted that one of the incentives for producing the claimed vectors and the packaging cells described in the '614 patent was the realization that many methods of *ex vivo* or *in vivo* gene therapy would not be compatible with the prolonged periods of selective culture used in previous methods of transduction (including the methods specifically described by Cone). In many respects, the above insight played a key role in motivating the development of retroviral vectors that lack a selectable

marker (i.e., the presently claimed vectors). Cone neither taught nor suggested the above insight.

7. I hereby further declare, under penalty of perjury under the laws of the United States of America, that all statements made herein of my own knowledge are true and that all statements made upon information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10/11/96

By: [signature]
Lawrence Cohen, Ph.D.

Exhibit 1

Curriculum Vitae
LAWRENCE K. COHEN, Ph.D.

PERSONAL:

Address:	5670 Cabot Drive Oakland, California 94611
Born:	April 19, 1953

EDUCATION

1974	B.A., Grinnell College Grinnell, Iowa
1976	M.S., University of Illinois Urbana, Illinois
1981	Ph.D., University of Illinois Urbana, Illinois

POSTDOCTORAL TRAINING:

1981-1983	RESEARCH FELLOW IN MICROBIOLOGY AND MOLECULAR GENETICS, Dana Farber Cancer Institute, Harvard Medical School, with Jack L. Strominger
1983-1985	RESEARCH FELLOW IN BIOLOGICAL CHEMISTRY, Harvard Medical School, with Bryan E. Roberts

AWARDS:

1981	American Cancer Society Fellowship
1982-1985	NIH, National Research Service Award
1985-1986	Medical Research Foundation Grant

PROFESSIONAL EXPERIENCE:

1993-present	VICE PRESIDENT, RESEARCH AND DEVELOPMENT Somatix Therapy Corporation Alameda, California
1992-1993	DIRECTOR, MOLECULAR BIOLOGY Somatix Therapy Corporation Alameda, California
1991-1992	MANAGER, MOLECULAR BIOLOGY Somatix Therapy Corporation Alameda, California
1988-1991	PROGRAM LEADER VASCULAR GRAFT PROGRAM Somatix Corporation Cambridge, Massachusetts
1987-1988	SENIOR SCIENTIST AND GROUP LEADER OF VIROLOGY & TISSUE CULTURE Applied bioTechnology, Inc. Cambridge, Massachusetts

1986-1987 **RESEARCH SCIENTIST**
Applied bioTechnology, Inc.
Cambridge, Massachusetts

1985-1986 **LECTURER IN BIOLOGICAL CHEMISTRY**
Harvard Medical School
Cambridge, Massachusetts

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High Efficiency Gene Transfer into Primary Human Tumor Explants without Cell Selection[1]

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Abstract

Preclinical studies with murine tumor models have demonstrated that autologous tumor cell vaccines engineered to secrete certain cytokines in a paracrine fashion elicit systemic immune responses capable of eliminating small amounts of established tumor. These results have engendered much interest in developing this strategy for gene therapy of human cancer. The major limitation to creating genetically modified autologous human tumor vaccines is efficient gene transfer into primary tumor explants, since the majority of human tumors fail to proliferate in long-term culture. Using the retroviral vector MFG in conjunction with short-term culture techniques, we have achieved, in the absence of selection, a mean transduction efficiency of 60% in primary renal, ovarian, and pancreatic tumor explants, and we have developed an autologous granulocyte-macrophage colony-stimulating factor secreting tumor vaccine for clinical trials.

Introduction

Recently, a new tumor vaccine approach using genetically altered autologous tumor cells to secrete local concentrations of cytokines has been developed in murine models (1–9). In some of these models, lymphokine gene transduced tumor cells have been shown to generate a local, tumor-specific immune response when administered as a s.c. vaccination (1–3, 5–9). In addition to rejecting the genetically modified tumor cells, vaccinated animals may develop a T-cell-dependent systemic immunity which in some cases can cure micrometastases established prior to treatment with the genetically altered tumor cells (1–3, 7, 8). Recently, a direct comparison of multiple cytokine genes transferred into a poorly immunological murine melanoma model identified GM-CSF[3] as the most potent in generating a protective response (7). Two critical features of this approach include: (*a*) the ability to generate a T-cell dependent tumor-specific systemic immunity; and (*b*) the production of lymphokine only at the tumor site, thereby producing a strong antitumor immune response without systemic toxicity.

The extension of this strategy to human cancer therapy will require two technical advances. First, the gene transfer systems used must be able to routinely introduce lymphokine genes into human tumors efficiently and must be able to produce consistent levels of gene expression. Other investigators report an efficiency of transduction of no better than 1 in 100 tumor cells so that the vector carrying the cytokine gene must also transfer a selection marker.[4] Second, the tumor cells that are transduced must be from primary human tumor cultures established at the time of surgery. All previous reports of gene transfer into human tumors have used vectors containing selectable markers and stable long-term cell lines rather than primary tumor cell cultures. Because stable long-term cultures cannot be established for the vast majority of human tumor explants (melanoma being the exception), these gene transfer approaches will fail to generate sufficient numbers of genetically modified cells (10, 11).[4] Even for the rare circumstances in which long-term cell lines can be established, transduction of cell lines and posttransduction selection might result in selective loss of expression of critical tumor-specific antigens expressed by the parent tumor *in vivo*. Boon *et al.* (12, 13) have provided evidence to support this contention by showing that it is possible to isolate several tumor-specific T-cell clones from a patient with malignant melanoma. Evaluation of these T-cell clones for lysis of melanoma tumor cell clones obtained from the same patient revealed three melanoma-specific antigens. The first antigen was present on all melanoma clones tested, the second antigen was lost during long-term culture, and the third antigen was expressed on a minority of tumor clones (12, 13).

In this article we report the use of a retroviral vector system to achieve high efficiency transduction of primary human tumor explants without requiring long-term culture or selection. These results provide the basis for the routine production of genetically modified autologous tumor vaccines.

Materials and Methods

Patients. All surgical specimens were obtained from patients with a histological diagnosis of either renal cell carcinoma; ovarian carcinoma; adenocarcinoma of the lung, colon, and pancreas; squamous cell carcinoma of the hypo pharynx; or carcinoma of the breast. All of the tumors were primary resections except for the five ovarian tumors which were obtained from ascites and the two breast carcinomas which were obtained from pleural fluid. Informed consent to use these surgical specimens was obtained from all patients prior to the surgical procedure.

Dissociation of Primary Human Tumor Explants. All tumors were transported from the operating room on ice and were mechanically dissociated into 1–5-mm fragments within 1 h. These tumor fragments were then enzymatically digested, initially by exposure to collagenase (GIBCO; 1 mg/ml; 173 units/mg), for 20–30 min in a vigorously shaking 37°C incubator. Single cells in the supernatant were removed. The remaining pellet was exposed to two more cycles of enzymatic digestion with collagenase followed by trypsin-EDTA (GIBCO; 0.25% trypsin, 1 mM EDTA) and DNase I (776785; 0.1 mg/ml; Boehringer Mannheim Biochemical) until all of the fragments were fully digested. This process yields approximately 5×10^6 viable malignant cells

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[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

[4] S. A. Rosenberg, W. F. Anderson, M. R. Blaese, S. E. Ettinghausen, P. Hwu, S. I. Karp, A. Kasid, J. J. Mule, D. R. Parkinson, J. C. Salo, D. J. Schwartzentruber, S. L. Topalian, J. S. Weber, J. R. Yanelli, J. C. Yang, and W. M. Linehan. Immunization of cancer patients using autologous cancer cells modified by insertion of the gene for interleukin-2, Recombinant DNA Advisory Committee Protocol, approved 3/1/92.

from a 1.0-g tumor mass. Cells from all digested fractions were pooled and incubated in selected growth medium. Cells were passaged when each flask reached 80–100% confluence.

***In Vitro* Growth of Primary Human Tumor Explants.** Conditions necessary for the short-term growth of primary human tumor cultures were evaluated in the following way. Freshly digested tumor cells were plated in duplicate at 1×10^5 cells/75 cm^2 flask. Each growth condition was evaluated both separately and in combination with other growth supplements (Tables 1 and 2). Different media including RPMI, Dulbecco's modified Eagle's medium, Ham's and Aim-V preparations, and lots of FBS were the initial components of growth medium screened (Tables 1 and 2). Following identification of the optimal medium and serum, additional additives were systematically evaluated (Table 2). Each supplement was evaluated for at least 2 *in vitro* passages/patient tumor and for enhancing the growth of at least 2 different patients' tumors of before routinely including it as a supplement for tumor growth. When tumor cells in each flask reached 100% confluence, they were trypsinized and counted before being replated.

Transduction of Primary Human Tumor Cultures. Transduction is performed with the MFG retroviral vector system. The structure of MFG has recently been described (7, 17). Briefly, in this vector, Moloney murine leukemia virus long terminal repeat sequences are used to generate both a full length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Moloney murine leukemia virus *env* mRNA) which is responsible for the expression of inserted sequences. The vector retains both sequences in the viral gag region shown to improve the encapsidation of viral RNA (16) and the normal 5′ and 3′ splice sites necessary for the generation of the *env* mRNA. Protein coding sequences are inserted between the *Nco*I and *Bam*HI sites in such a way that the initiation codon of the inserted sequences is placed precisely at the position of the viral *env* initiation codon, and minimal 3′ nontranslated sequences are included in the insert. No selectable marker exists in the vector. Complementary DNA sequences encoding the cytokine were inserted into MFG and the resulting vector constructs were introduced into CRIP cells as previously described (17) in order to generate recombinant virus with amphotropic host range.

The retroviral producer cell line, CRIP, is grown in culture to confluency in Dulbecco's modified Eagle's medium + 10% calf serum. Two days prior to transduction, the cells are trypsinized and replated at a density of 2×10^6 cells/100-mm culture dish. One day prior to transduction, 10 cc of fresh medium are added to the cells. On the day of transduction, a 24-h supernatant is collected and filtered through an 0.45-μm filter to remove contaminating retroviral producer cells. Human tumor explants growing in culture at a density of 5×10^5 cells/75 cm^2 culture dish are incubated with 10 ml of retroviral supernatant containing 70 μg/ml of DEAE-dextran (Sigma, St. Louis, MO; D-9885) at 37°C. (Of note, it is important to perform a dose response curve to compare new preparations of DEAE-dextran prior to use because we have found that the optimal concentration of DEAE-dextran needed for successful transduction can vary between preparations.) A 4-h incubation period is optimal for efficient gene transfer. Following transduction, the retroviral supernatant is removed and the cells are grown in culture for an additional 3 days to allow for integration and expression of the transferred DNA.

Assay for *LacZ* Expression. To determine the rate of transduction of primary human tumor explants using the MFG retroviral vector system, we used a vector containing the *LacZ* marker gene. This gene encodes for expression of *Escherichia coli* β-galactosidase and is used because it is easy to assay for its expression using cytochemistry methods (18).

Three days after infection with the retroviral vector, adherent tumor cells are trypsinized and washed with PBS (pH 7.3). The tumor cells are then fixed with gluteraldehyde (final concentration of 0.5% gluteraldehyde in PBS) for 5 min on ice. The cells are subsequently washed once with PBS and resuspended in a substrate solution containing the substrate bluogal at a final concentration of 300 μg/ml, 0.2% of a 1 M solution of $MgCl_2$, 0.16% potassium ferricyanide, and 0.2% potassium ferrocyanide in PBS (pH 7.3). The tumor cells are incubated overnight at 37°C without CO_2. Following incubation, the tumor cells are counted on a hemocytometer. The transduction efficiency is defined as the percentage of positively stained cells. At least 200 cells are counted/specimen. Cells are determined to be positive for the transferred gene if they have a cytoplasm that appears to be uniformly blue.

Assay for Human GM-CSF Production. The TF-1 cell line, described by Kitamura *et al.* (19), is a human line isolated from a patient with erythroleukemia and is used to evaluate GM-CSF production by transduced human tumor cultures. Growth of these cells is dependent on the addition of GM-CSF to their media. These cells are passaged 3 times/week in RPMI 1640 supplemented with 10% FBS, 1 mM L-glutamine, 50 μg/ml penicillin and streptomycin, and 5 ng/ml of recombinant human GM-CSF. A [^{3}H]thymidine incorporation proliferation assay using these cells is performed by first washing these cells 3 times to remove GM-CSF. The experimental supernatants obtained by growing 1×10^5 transduced human tumor cells for 24 h in 5 cc of tumor growth medium are collected and filtered through an 0.45-μm filter to remove contaminating cells and plated (100 μl/well) in 96-well flat plates at 1:3 dilutions. 1×10^4 TF-1 cells (100 μl/well) are added to each well in growth medium without GM-CSF and are incubated × 24 h at 37°C.

[^{3}H]thymidine (1 μCi) is added to each well and the cells are incubated for an additional 18–24 h. At the end of the incubation period the cells in each well are harvested and counted in a beta counter.

Results

Establishing Primary Human Tumor Cultures. Our method of digesting freshly excised tumor specimens routinely yields 5×10^6 viable tumor cells/g of excised tumor. It should be pointed out that

Table 1 *Basal media and FBS screened for primary human tumor growth support in vitro*

At least 2 histologically similar tumor cell types were grown in each of the basal media listed above and studied for enhanced *in vitro* proliferation. Varying amounts of FBS were also evaluated. Growth rate was initially evaluated by daily observation and scoring of duplicate flasks.[a]

Medium	Renal cell carcinoma	Ovarian carcinoma	Pancreatic and colonic carcinoma	Breast carcinoma	Adeno lung and SCCA[b,c]
Base medium					
RPMI 1640	++[c]	++	++	–	++
Ham's F10	++	++	NT	–	NT
Modified Eagle's	–	–	NT	NT	NT
Aim-V	–	–	NT	–	NT
DMEM[d] Ham's F12	NT	NT	NT	++	NT
Defined FBS (%)					
0	–	–	–	–	NT
5	–	–	–	+++	NT
10	–	–	–	++	NT
15	++	++	++	++	NT
20	++++	++++	++++	NC	++++
25	++	++	NT	NT	NT

[a] Cultures were scored using the following scoring system: NC, no change in growth rate; –, decreased growth; ++, 2-fold increase in growth; +++, 3-fold increase in growth; ++++, 4-fold increase in growth; NT = not tested (a 2-fold increase in growth = 2 [times] the number of cells obtained from the control flask during a 96-h incubation period). When the cells in each flask reached 100% confluence (total cell number = approximately 2×10^6/flask), they were trypsinized, counted, and replated for further growth evaluation. The number of passages reached/given time period was also recorded. For some tumors the doubling time was estimated using a [^{3}H]thymidine uptake proliferation assay.

[b] Adenocarcinoma (Adeno) of the lung and squamous cell carcinoma (SCCA) of the tonsil.

[c] Only one tumor of each tested.

[d] DMEM, Dulbecco's modified Eagle's medium.

Table 2 Growth supplements screened for primary human tumor growth support *in vitro*

• At least 2 histologically similar tumor cell types were studied for enhanced *in vitro* proliferation using each of the growth supplements listed above. All supplements were added to the basal medium and FBS selected for optimal tumor growth (Table 1). Growth rates of duplicate flasks of tumor cells were evaluated daily using the scoring system described in the methods section and in Table 1. When the cells in each flask reached 100% confluence, they were trypsinized, counted, and replated for further growth evaluation. The number of passages reached/given time period was also recorded (data not shown). For some tumors the doubling time was estimated using a [^{3}H]thymidine uptake proliferation assay.

Growth supplement	Renal cell carcinoma	Ovarian carcinoma	Pancreatic and colonic carcinoma	Breast carcinoma	Adeno lung and SCCA[b,c]
Recombinant GF[c]					
Human insulin	NC	++++	++++	++	++
Epidermal GF	++	++	NC	++	++++
Fibroblast GF	NC	NC	NT	NT	NT
Insulin-like GF-1	NC	++	NC	NC	NC
Insulin-like GF-2	NC	NC	NC	NC	NC
IL-6	NC	NC	NT	NT	NT
IL-3	NC	NC	NT	NT	NT
Keratinocyte GF	++	++	NT	NT	NT
Insulin-like GF 1 + 2	++	++	++	NT	NT
Keratinocyte + Epidermal GF	++	+++	NT	NT	NT
Other supplements					
Hepatocyte GF	+++	NC	++	NT	NT
BPE	++	NC	++	++	NT
Hydrocortisone	++	NC	++	++	NT
Ascorbic acid	NT	NT	–	++	NT
Keratinocyte + Hepatocyte GF	++	NC	++	NT	NT
Matrigel	NC	NC	NT	NT	NT
Selenium + Transferrin	NC	+++	+++	+++	+++
Triiodothyronine	NT	NT	–	++++	NT
Glucagon					
Estradiol					
Ethanolamine					
Phosphoethanolamine (14)					
TPB (15)	++++	NC	NT	NT	NT

[a] Adenocarcinoma (Adeno) of the lung and squamous cell carcinoma (SCCA) of the tonsil.
[b] Only one tumor of each tested.
[c] GF, growth factors; BPE, bovine pituitary extract; TPB, tryptose phosphate broth.

these results take into account our recent data which suggest that mechanical dissociation into 5-mm tumor fragments prior to digestion is superior to enzymatically digesting smaller tumor fragments, especially when only collagenase is used for the initial enzymatic digestion.[5] In fact, when tumor cells that are mechanically dissociated are grown separately from collagenase-digested tumor cells but in the same growth medium, the initial growth rate of the mechanically dissociated tumor cell population is much slower, resulting in roughly one-half the number of expanded cells during the first two *in vitro* passages (data not shown).

We have evaluated 24 renal cell carcinomas, 26 ovarian carcinomas, 8 colon carcinomas, 5 pancreatic carcinomas, 3 breast carcinomas, an adenocarcinoma of the lung, and a squamous cell carcinoma of the tonsil for short-term *in vitro* growth. Growth conditions were studied using the procedure described in "Materials and Methods." Initially, the optimal base medium and FBS were determined for each histological tumor type (Table 1). Lots of characterized and defined FBS were screened. Once an adequate lot of serum was identified, the percentage of FBS was evaluated. A list of the base media and percentage of defined fetal bovine sera is shown in Table 1. Additional supplements were subsequently evaluated. A list of these growth supplements can be found in Table 2. Optimal short-term growth of fresh human tumor explants is dependent on several conditions. Common to all histological tumor types with the exception of breast carcinoma is the percentage of high grade FBS used in the buffered medium. All of the other tumors grown in our laboratory to date grow well in 20% characterized or defined FBS. Breast carcinomas require 5% FBS or less. Greater than 5% FBS can result in overgrowth of fibroblasts (14). Ovarian, colon, and pancreatic tumor explants also require the addition of human insulin (0.2 units/ml). The addition of transferrin and selenium will often enhance the growth of ovarian and breast carcinomas. Renal cell carcinoma explants require the addition of tryptose-phosphate broth (10%; Difco; 0060–01–6) and occasionally, bovine pituitary extract (Sigma; P1167). Colon tumor explants grow well in a medium that is also supplemented with bovine pituitary extract. With these defined conditions an expansion of the tumor cell population of 10-fold or greater is routinely obtained during a period of 2–4 weeks (Table 3).

In addition, an attempt was made to identify characteristics of the initial tumor specimen that were associated with enhanced or inhibited *in vitro* growth. In particular, histological diagnosis, degree of malignant cell differentiation, and degree of necrosis were compared with the last *in vitro* passage achieved by the tumor. Interestingly, only the degree of necrosis adversely affected the success of short-term *in vitro* growth. In contrast, all malignant histologies could be grown equally well provided that the conditions for each histology were optimized. To illustrate this point, the results for 26 nephrectomy specimens evaluated for *in vitro* expansion are shown in Table 3.

Transduction of Human Tumor Explants. We have identified three conditions that are critical for high efficiency gene transfer to primary cultures of human tumor cells. First, successful tranduction requires a vector system that can transduce cells efficiently, resulting in consistent levels of gene expression. Although the quality of retroviral supernatants can vary, this problem is easily controlled by tittering of the retroviral supernatants using easily transducible cell lines prior to use in gene transfer to the fresh human tumor explants. Second, efficient retroviral gene transfer and expression depends on the percentage of tumor cells within the tumor population that are actively proliferating at the time of gene transfer. In general, transduction efficiency correlates with the percentage of tumor cells undergoing cell cycling since integration of the retroviral vector into the host genome is required for expression of the transferred gene. Third,

[5] A. Burns, L. Cohen, R. C. Donehower, G. Dranoff, K. M. Hauda, E. M. Jaffee, A. J. Lazenby, H. I. Levitsky, F. F. Marshall, R. C. Mulligan, W. G. Nelson, A. H. Owens, D. M. Pardoll, G. Parry, A. H. Partin, S. Piantadosi, J. W. Simons, and J. R. Zabora. Phase I study of non-replicating autologous tumor cell injections using cells prepared with or without GM-CSF gene transduction in patients with metastatic renal cell carcinoma. Recombinant DNA Advisory Committee Protocol, approved 3/1/93.

Table 3 *Results of in vitro expansion of 26 fresh human renal cell explants after nephrectomy*

All tumors were obtained at the time of surgical excision; mechanically dissociated; and enzymatically digested into a single cell suspension. Cells were grown *in vitro* using the growth conditions described in "Results." The initial cell number was usually 1×10^7 cells obtained from a 2-g tumor mass. Tumor cells were passaged every 4–5 days by trypsinizing the cells off of the culture flask and splitting them 1:3. A passage (P) was defined as the point at which the tumor cells reached 100% confluence. The *n*-fold increase represents the estimated increase in tumor cells if the total number of cells obtained at the end of each passage were continued in culture until the last passage obtained (3-fold increase = 3 100% confluent tissue culture flasks obtained as the result of splitting a single flask 1:3). The number of patient specimens (N) with a particular histological subtype that reached each *in vitro* passage is also recorded in parentheses.

Last passage	*n*-fold increase	Histological subtype (N)
P2	9	Oncocytoma (1) Clear cell, grade III (1)[a]
P3	27	Oncocytoma (2) Papillary, grade II (1)[b]
P4	81	Granular, papillary (3) Granular (1) Grade I, clear cell (1) Grade II, clear cell (1) Grade III, clear cell (1)
P5	243	Sarcomatoid (1) Grade II, clear cell (1) Grade III, multicystic (1) Papillary, grade II (1)
P6	729	Grade II, clear cell (2)
P7	2187	Grade II, clear cell (3) Papillary, clear cell (1) Sarcomatoid (1)
No growth	0	Benign histologies (2) Oncocytoma (1)

[a] Small tumor, too few cells obtained.
[b] Severe necrosis.

transduction efficiency can be enhanced by the addition of polymers to the retroviral supernatant just prior to exposure of the target cells to the retroviral vector. Enhanced gene transfer is thought to occur via a charge-mediated mechanism that affects virus binding to or penetration of the target cell. The polycations protamine, polybrene, and DEAE-dextran are routinely used for this purpose (20).[4] However, protamine, which has been commonly used for previous gene therapy trials, gives low efficiencies of gene transfer to human lymphocytes and tumor cell lines, and therefore requires the cotransfer of a selectable marker.[4] We therefore performed an extensive comparison of polybrene and DEAE-dextran, using a wide range of concentrations and exposure times of these agents to the target cell. Although both agents increased the transduction efficiency of human tumor cells in primary culture, DEAE-dextran resulted in less cell toxicity than polybrene at equivalent enhancement doses. Thus, we find that DEAE-dextran is a potent enhancer of gene transfer to primary human tumor cultures. It also results in less target cell toxicity.

An ovarian tumor cell line derived from a patient with malignant ascites was initially used to evaluate optimal transduction conditions. These conditions are described in "Materials and Methods."

These conditions were then used to evaluate 8 renal cell carcinomas, 5 ovarian carcinomas, 5 colorectal carcinomas, 2 pancreatic carcinomas, 2 breast tumors, 1 squamous cell carcinoma of the tonsil, and 1 adenocarcinoma of the lung for transduction efficiency using the MFG vector carrying the *E. coli LacZ* gene (Fig. 1*A*). Using the conditions described above, we were able to show expression of the *LacZ* marker gene in at least 20% of each tumor population, with a mean of 70% for renal cell carcinomas, 65% for ovarian carcinomas, and 43% for colon carcinomas. In 16 of 21 tumors, we were able to achieve at least a 40% transduction efficiency. We have also attempted to transduce several other histological human tumors, including a squamous cell carcinoma arising from a tonsil (40% transduction), an adenocarcinoma of the lung (82% transduction), and 2 breast carcinomas (mean transduction efficiency of 28%).

Recent studies evaluating the antitumor immune response generated by a variety of single lymphokines using the B16 melanoma murine model revealed that the cytokine GM-CSF can generate an enhanced antitumor immune response that is much greater than the response generated by any other cytokine tested (7). *In vivo* depletion studies revealed that this response is dependent on both CD4+ and CD8+ T-cells. Additional experiments indicated that maximal systemic immunity was achieved when the average level of GM-CSF production by the vaccine cells was equal to or greater than 36 ng/10^6 cells/24 h.[5] These preclinical studies provide the immunological data needed to begin to apply this approach to the treatment of cancer in patients. As a prelude to initiating clinical vaccine trials, we used the same MFG retroviral vector to transfer the human GM-CSF gene to 3 renal cell carcinomas, 2 colon carcinomas, and 3 pancreatic carcinomas (Fig. 1*B*). In 6 of the 8 tumors, GM-CSF production was at least 50 ng/10^6 cells/24 h. It was even possible to improve production of GM-CSF by the less efficiently transduced tumors, 1 to more than 50 ng/10^6 cells/24 h, after a second retroviral transduction was performed. Southern blot analysis of 5 of the renal cell carcinoma cultures genetically altered to secrete GM-CSF revealed a range of integrated vector copy numbers between 0.5 and 2 copies/cell. This correlated with a range of GM-CSF secretion between 26 and 74 ng/10^6 cells/24 h (Table 4). In addition, the transduced cells can freeze and thaw easily, with minimal loss of the number of viable, lymphokine-producing cells (Fig. 1*B*). This confirms that the MFG vector system has the ability to very efficiently transfer human cytokine genes to fresh human tumor explants.

Discussion

Preclinical murine studies have shown that tumor cells, genetically altered to secrete lymphokines, will increase the immunogenicity of a tumor when given s.c. in the form of a vaccine. The gene transfer of GM-CSF, in particular, stands out as the cytokine that generates the greatest antitumor immune response in murine models (7). Furthermore, the local secretion of GM-CSF at the site of the tumor leads to the production of both helper and cytolytic cells that can circulate and eradicate existing tumor at distant sites. In addition, our previous studies have also shown that tumor cells genetically altered to secrete local concentrations of GM-CSF will cure mice of micrometastatic melanoma. These studies therefore provide the theoretical basis for using this approach to treat human cancers.

We now report that it is technically possible to produce a genetically-altered autologous human tumor vaccine for patient trials. In addition, we provide evidence for successful gene transfer to short-term, primary tumor cultures, which is a critical advantage over previous reports of genetically altered long-term human tumor cell lines. Since the goal of a genetically altered tumor vaccine is to activate the immune system of a patient to recognize and eradicate existing tumor at other sites, therapeutic efficacy will depend on reinjecting a population of vaccine cells that represent the antigenic diversity of the parent population. There is now evidence that suggests that long-term *in vitro* culture of human tumor cell lines results in the loss of expression of relevant tumor antigens. For example, Boon *et al.* (12, 13) found that the immunodominant T-cell recognized antigen in a human melanoma was spontaneously lost upon long-term culture and subcloning. To our knowledge, this is the first report of high efficiency gene transfer to primary human tumor cultures without requiring simultaneous transfer of a selection marker for *in vitro* selection of the transduced cells.

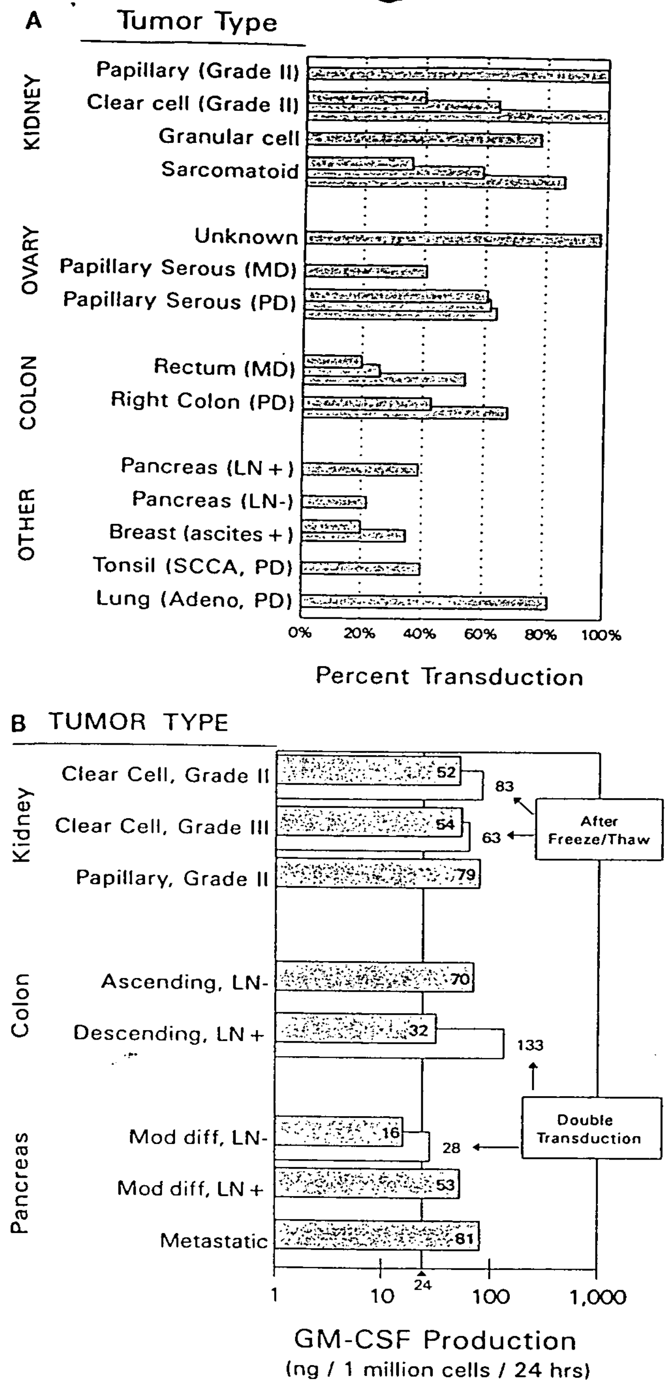

Fig. 1. *A*, transduction efficiency of primary human tumor cell populations. All tumor cells were transduced with the MFG retroviral vector carrying the *E. coli LacZ* marker gene. β-galactosidase activity was assayed using the substrate staining procedure described in "Materials and Methods." Percentage transduction was calculated as the total number of positively stained cells/200 cells stained. *MD*, moderately differentiated; *PD*, poorly differentiated; *LN*, lymph node; *SCCA*, squamous cell carcinoma; *Adeno*, adenocarcinoma. *B*, GM-CSF production by transduced renal cell, colonic, and pancreatic tumor cells. All tumor cells were transduced with the MFG retroviral vector carrying the human GM-CSF gene. GM-CSF production by 1 million transduced tumor cells over a 24-h period at least 3 days following transduction was determined using the bioassay described in "Materials and Methods." Two transduced renal tumor cell cultures were evaluated for GM-CSF production before and after freezing and thawing the tumor cells. One pancreatic and two colon tumor cultures were evaluated for GM-CSF production after two consecutive GM-CSF gene transfers. The second GM-CSF gene transfer was performed 72 h following the first gene transfer. Of note, GM-CSF production by primary untransduced tumor cultures at the corresponding *in vitro* passage was also determined. GM-CSF production was detected by 2 renal cell carcinomas (12 *versus* 83 and 3.2 *versus* 78.6 ng/10^6 cells/24 h, untransduced *versus* transduced, respectively), and 1 colonic carcinoma (20 *versus* 70 ng/10^6 cells/24 h, untransduced *versus* transduced, respectively). *LN*, lymph node. *N*-(2,3-Dioleoyloxy)propyl)-*N,N,N*-trimethyl-ammoniummethylsulfate, a cationic lipid often used for transfection of DNA into mammalian cells (available from Boehringer Mannheim), was used instead of DEAE-dextran to enhance retroviral infection. It was found to be as effective but slightly less toxic to the tumor cells when compared with DEAE-dextran, enabling a second transduction to be performed on the same cells. Although the second transduction resulted in improved GM-CSF production, its cost may prohibit its use in clinical trials.

Table 4 *Results of the comparison of GM-CSF secretion with vector copy number in GM-CSF transduced human renal cell tumor cultures*

Five primary renal cell tumor cultures were plated at 3.5×10^6 cells in 225-cm^2 tissue culture flasks and transduced 24 h later with 25 ml of retrovirus containing supernatant in the presence of 10 μg/ml DEAE-dextran for 24 h. GM-CSF secretion was determined by enzyme-linked immunosorbent assay 48 h later and all values were normalized to ng/10^6 cells/24 h. The concentration of GM-CSF secretion was also determined for untransduced human renal carcinoma cultures at the same *in vitro* passage number. Vector copy number was determined by Southern blot analysis.

GM-CSF secretion pretransduction	GM-CSF secretion posttransduction	Vector copy no.	Histological subytpe
6.4	35	1.6	Grade II, clear cell
1.2	26	0.5	Grade II and III, clear cell and granular
2.5	48	2.0	Grade II, clear cell
3.4	74	0.9	Grade I with oncocytic features
4.7	63	0.7	Grade II with oncocytic features

A proliferating population of primary human tumor cells is critical for retroviral mediated transduction since proliferation of the majority of tumor cells within the culture is necessary to facilitate integration. For this reason, growth conditions necessary for *in vitro* expansion of several histologically different fresh human tumor explants were optimized. It is also worth noting that even the short-term culture of the primary tumor cells yielded a significant increase (greater than 10-fold) in the total number of tumor cells. In fact, of 26 fresh human renal cell explants received after nephrectomy, 21 of the 24 specimens (88%) with malignant histological subtypes were propagated in culture long enough to successfully undergo gene transfer.

Murine tumor vaccine studies have revealed that for GM-CSF a full antitumor immunization potential is obtained over a greater than 10-fold range of cytokine concentrations (7). However, immunization potential was extremely dependent on vaccinating cell dose, with increasing doses providing increased systemic protection against tumor challenge. It is therefore likely that at least 1×10^8 cytokine-secreting tumor cells will be needed to generate an optimal antitumor immune response in patients. Thus, to produce tumor vaccines from the majority of patient specimens, either 10 g of viable tumor must be available or the primary culture must be expanded at least 10-fold. Since the average weight of excised tumor specimens received by our laboratory is 2–3 g, in most cases vaccine development will depend on the success of *in vitro* expansion. Given that the majority of tumor cells proliferate for 2–3 passages under the growth conditions we have developed, it is unlikely that major populations of antigen-bearing cells will be selected out during the short-term culture period.

Our system has two further advantages over past approaches. First, we have found that it is possible to freeze and thaw previously transduced tumor cells without loss of cell viability and gene expression. This should allow for flexibility in therapy administration. Second, these cells can be irradiated following transduction, resulting in the inhibition of cell proliferation without loss of *in vitro* GM-CSF production. Our preclinical animal studies confirm the *in vivo* efficacy of these irradiated tumor vaccines (7). Thus, this vaccine should be as safe as it is effective.

The transduction efficiency ranged from 39 to 100%. This was particularly true when the vector was used to transfer the *LacZ* marker

gene. There are at least three possible explanations for variability. First, successful gene transfer may be dependent on the histological tumor type or the degree of cellular differentiation of the tumor cells that are being tranduced. Our data do not demonstrate a correlation of transduction efficiency with the histological cell type or degree of differentiation of the renal cell, pancreatic, and colon carcinomas that have been evaluated so far. In contrast, all of the ovarian carcinomas were from ascites, which may explain the less variable range of transduction efficiency among these more advanced populations of tumor cells. A comparison could not be made for the breast, lung, and squamous cell carcinomas because too few tumors of these histological types were evaluated. Second, integration of the transferred gene is dependent on proliferation of the tumor cell population. Therefore, efficient gene transfer requires proliferation of the majority of tumor cells within the explanted population. It may be that suboptimal tumor cell proliferation of some primary tumor cultures explains the wide range of transduction efficiencies among the initial gene transfer experiments performed using the *LacZ* marker gene. However, it does not account for the entire problem since we see less variation in transduction efficiency with improvement of our technique of retroviral supernatant collection. Third, transduction efficiency is dependent on the virus titer of retroviral supernatant, which will vary with different titer collections. This should no longer represent a significant practical problem as recent advances in long-term freezing of amphotropic retroviral supernatants will allow lots to be tested for titer prior to their use in vaccine preparation.[5]

In addition, when GM-CSF secretion was compared with the vector copy number for 5 genetically altered renal cell cultures, the number of integrated copies/cell did not perfectly correlate with the concentration of GM-CSF produced. A possible explanation for this is that expression of retrovirally transferred genes may be dependent on host cell-derived transcription factors that vary among different histologically similar tumor cultures. We also noted that with increasing passage of the human renal tumors cells, endogenous GM-CSF production was induced. However, levels of endogenous GM-CSF (which ranged from 0 to 20 ng/10^6 cells/24 h, with an average of 4.1 ng/10^6 cells/24 h) were far below the threshold for maximal vaccine potency (36 ng/10^6 cells/24 h) as determined in our animal studies.

In conclusion, we have shown that it is possible to establish and efficiently transduce short-term, primary human tumor cultures. The MFG retroviral vector system has made it technically feasible to provide safe, efficient gene therapy to patients with cancer. In addition, this vector appears flexible enough to transduce a wide variety of histological tumor types. We are planning to use this vector system in a Phase I study to evaluate the antitumor immune response generated by autologous GM-CSF-secreting renal tumor cells in patients in the near future.

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